ApoGSH™ Glutathione Colorimetric Detection Kit

(Catalog #K261-100; 100 assays; Store kit at -20˚C)

I. Introduction:

Glutathione (GSH) is the major intracellular low-molecular-weight thiol that plays a critical role in the cellular defense against oxidative stress in mammalian cells. BioVision's ***ApoGSH™ Glutathione Colorimetric Assay Kit*** provides a convenient, colorimetric method for analyzing either total glutathione or the reduced form glutathione alone using a microtiter plate reader. The assay is based on the glutathione recycling system by DTNB and glutathione reductase (Fig. 1). DTNB and glutathione (GSH) react to generate 2-nitro-5-thiobenzoic acid which has yellow color. Therefore, GSH concentration can be determined by measuring absorbance at 412 nm. The generated GSSG can be reduced back to GSH by glutathione reductase, and GSH reacts with DTNB again to produce more 2-nitro-5-thiobenzoic acid. Therefore, the recycling system dramatically improves the sensitivity of total glutathione detection. The kit includes the 5-Sulfosalicylic acid (SSA) for the removal of proteins from samples and for the protection of GSH oxidation and γ-glutamyl transpeptidase reaction. The kit can quantify glutathione from 1-100 ng/well in a 200 □l reaction. For detecting lower glutathione concentrations, such as in blood samples, increasing reaction time will generate stronger signal. The kit can also specifically detect the reduced form of glutathione (GSH) by omitting the glutathione reductase from the reaction mixture. The sensitivity for detecting the reduced form of glutathione (without recycling system) is 100 times lower than detecting the total glutathione.

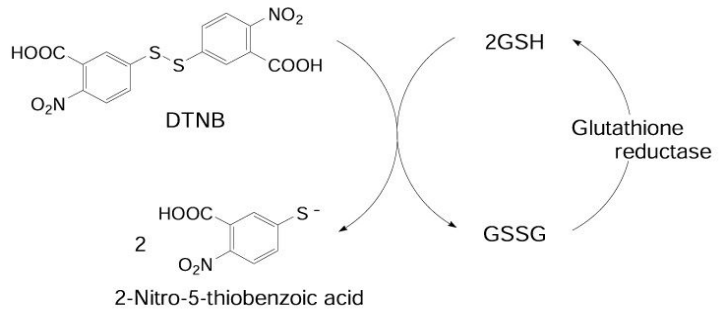

Fig. 1. Principle of Total Glutathione Assay.

II. Kit Contents:

Component	K261-100	Color code	Part
	100 assays	Cap color	Number
Glutathione Reaction Buffer	100 ml	NM	K261-100-1
Glutathione Substrate (DTNB)	2 vials	Red	K261-100-2
NADPH Generating Mix (lyophilized)	2 vials	Blue	K261-100-3
Glutathione Reductase (lyophilized)	2 vials	Green	K261-100-4
Sulfosalicylic Acid (SSA, 1 gram)	1 bottle	WM	K261-100-5
GSH Standard (lyophilized, MW 307)	2 x 1 mg	Yellow	K261-100-6

III. Sample Preparation:

Note: Peptide thiol may interfere with the assay of reduced form glutathione. SSA treatment may not able to complete remove all small peptides from samples. Further purification may be required to accurately measure reduced form glutathione. Peptide thiols don't significantly interfere with total glutathione assay.

A. Cell Sample Preparation (0.5-1 x 10^6 cells/assay)

1. Treat cells by desired method. Concurrently incubate a control culture without treatment.
2. Collect cells by centrifugation at 700 x g for 5 minutes at 4˚C. Remove supernatant.
3. Resuspend cell pellet in 0.5 ml ice-cold PBS. Transfer into a 1.5 ml microcentrifuge tube, and centrifuge at 700 x g for 5 minutes at 4˚C. Remove supernatant.
4. Lyse cells in 80 µl ice-cold Glutathione Buffer. Incubate on ice for 10 minutes.
5. Add 20 µl of 5% SSA (see below for SSA preparation), mix well and centrifuge at 8000 x g for 10 min. Transfer supernatant to a fresh tube and use it for glutathione assay.

B. Tissue Sample Preparation (100 mg)

1. Homogenize the tissue in 0.4 ml of Glutathione Buffer.
2. Add 100 µl of 5% SSA (see below for SSA preparation), mix well, and centrifuge at 8000 x g for 10 minutes.
3. Transfer supernatant to a fresh tube and use it for glutathione assay.

C. Plasma Sample Preparation

1. Centrifuge an anticoagulant treated blood at 1000 x g for 10 min at 4˚C.
2. Transfer the top plasma layer to a new tube and add 1/4 vol of 5 % SSA. Mix well.
3. Centrifuge at 8000 x g for 10 min at 4˚C.
3. Transfer supernatant to a new tube, and use it for the glutathione assay.

D. Erythrocyte Sample Preparation

1. Centrifuge an anticoagulant treated blood at 1000 x g for 10 min at 4˚C.
2. Discard the supernatant and the white buffy layer.
3. Lyse the erythrocytes with 4 vol of Glutathione Buffer. Keep on ice for 10 min.
4. Add 1 vol 5% SSA, mix well, and centrifuge at 8000 x g for 10 minutes. Transfer supernatant to a fresh tube and use it for glutathione assay.

   Note: Erythrocytes can be isolated from the remaining sample solution after the plasma sample isolation.

IV. Preparation of Solutions & Storage Conditions:

Substrate: Add 1 ml of Glutathione Buffer to 1 vial of substrate and dissolve it completely. Store the remaining solution at –20˚C, stable for 2 months.

NADPH Generating Mix: Add 1 ml of Glutathione Buffer to 1 vial of the NADPH mix. Store the solution at -20˚C, stable for 2 months.

Glutathione Reductase: Add 1 ml of Glutathione Buffer to 1 vial of the enzyme and dissolve. Use up the solution within 1 day.

SSA: Add 19 ml of dH_2O to make 5% solution and then dilute 5 ml of the solution with Glutathione Buffer to make 1% SSA solution. Store at 4˚C, stable for 6 months.

GSH Standard: Add 1 ml of 1% SSA to the GSH standard vial to generate 1µg/µl GSH standard solution. Store at –20˚C, stable for 2 months.

V. Preparation of Solutions for Standard Curve:

To generate standard curve for detecting the reduced form of glutathione only, add 50, 40, 30, 20, 10, and 0 µl of the 1 µg/µl GSH standard into each labeled microcentrifuge tubes, add 1% SSA to make up for a total volume of 100 µl/tube.

To generate standard curve for detecting the total glutathione, dilute the 1 µg/µl glutathione solution into 10 ng/µl with 1% SSA. Add 50, 40, 30, 20, 10, and 0 µl of the 10 ng/µl GSH standard into each labeled microcentrifuge tubes, add 1% SSA to make up for a total volume of 100 µl/tube.

VI. Glutathione Assay Protocol:

1. Prepare enough Reaction Mix for the standard and samples to be assayed in 96-well plate (not provided). Each well should contain:

20 µl	NADPH Generating Mix
20 µl	Glutathione Reductase*
120 µl	Glutathione Reaction Buffer

*For detecting the reduced form of glutathione only, omit Glutathione Reductase. Use 20 µl of the Glutathione Reaction Buffer replace the 20 µl of glutahione Reductase.

2. Mix well. Add 160 µl of the Reaction Mix to each well and incubate at room temperature for 10 minutes to generate NADPH.
3. Add 20 µl of either the GSH standard solutions or the sample solution. Incubate the plate at room temperature for 5-10 min.

   Note: We recommend to make several dilutions of your sample using the 1% SSA to make sure the readings are within the range of the standard calibration curve.
4. Add 20 µl of Substrate solution, and incubate at room temperature for 5-10 min, or longer if the samples contain low levels of glutathione.

   Notes: a) Since the reaction starts immediately after the addition of substrate, use a multichannel pipette or repeating pipette is recommended to avoid the reaction time lag among wells.

   b) You can read samples immediately and at various times following addition of the substrate solution for kinetic studies.
4. Read the absorbance at 405 nm or 415 nm using a microplate reader.
5. Determine concentrations of GSH in the sample solutions using the standard glutathione calibration curve.

   Note: A. Using reduced form glutathione Standard Curve for detecting reduced form of glutathione. Using total Glutathione Standard Curve for detecting total glutathione. There are about 10 to 100 fold difference in detection sensitivity between detecting reduced form glutathione and total glutathione (see procedure step IV for preparation of standard curve).

   B. The colorimetric reaction is stable and the O.D. increases linearly over 30 min for total glutathione detection.

VII. Calculation of Total Glutathione

Pseudo-end point method: Total Glutathione = $(O.D._{sample} - O.D._{blank})$

Kinetic method: Total Glutathione = $(Slope_{sample} - Slope_{blank})/Slope$

VIII. Reagent Interference

Reducing agents such as ascorbic acid, β-mercaptoethanol, dithiothreitol (DTT) and cysteine, or thiol reactive compounds such as maleimide compounds, interfere with the glutathione assay and therefore should be avoided during the sample preparation.

When detecting the reduced form of glutathione, protein thiols can generate significant background signal. In such cases, it is necessary to completely remove proteins from samples. We suggest using Centrifugal Spin column with 10 kDa molecular weight cut off filter (*BioVision*, Cat. No. 1997-25) to remove proteins. Then the reduced glutathione can be easily detected from spin through samples.

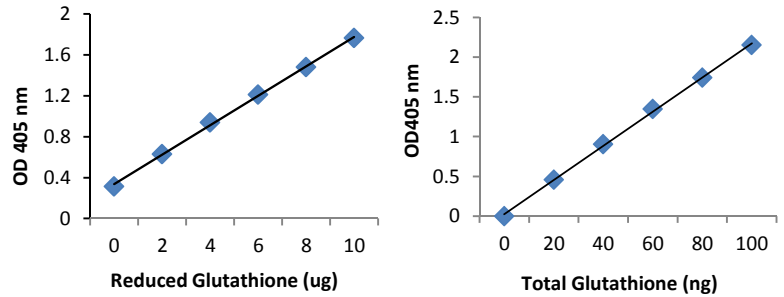

Fig .2. Glutathione Standard Curve. Various amounts of standard glutathione was added to the glutathione reaction and incubated for 10 min according to the kit instructions. Absorbance was measured at O.D. 405 nm.

RELATED PRODUCTS:

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- Mitochondrial Apoptosis Kits & Reagents
- Nuclear Apoptosis Kits & Reagents
- Apoptosis siRNA Vectors

Cell Fractionation System
- Mitochondria/Cytosol Fractionation Kit
- Nuclear/Cytosol Fractionation Kit
- Membrane Protein Extraction Kit
- Cytosol/Particulate Rapid Separation Kit
- Mammalian Cell Extraction Kit
- FractionPREP Fractionation System

Cell Damage & Repair
- HDAC Fluorometric & Colorimetric Assays & Drug Discovery Kits
- HAT Colorimetric Assay Kit & Reagents
- DNA Damage Quantification Kit
- Glutathione Fluorometric & Colorimetric Assay Kits
- Nitric Oxide Fluorometric & Colorimetric Assay Kits

Signal Transduction
- cAmp & cGMP Assay Kits
- Akt & JNK Activity Assay Kits
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Adipocyte & Lipid Transfer
- Recombinant Adiponectin, Survivin, & Leptin
- CETP Activity Assay & Drug Discovery Kits
- Total Cholesterol Quantification Kit

Molecular Biology & Reporter Assays
- siRNA Vectors
- Cloning Insert Quick Screening Kit
- Mitochondrial & Genomic DNA Isolation Kits

FOR RESEARCH USE ONLY! Not to be used on humans.

GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	• Use of ice-cold reaction buffer	• Reaction buffer must be at room temperature
	• Omission of a step in the protocol	• Refer and follow the data sheet precisely
	• Plate read at incorrect wavelength	• Check the wavelength in the data sheet and the filter settings of the instrument
	• Use of a different 96-well plate	• Fluorescence: Black plates ; Luminescence: White plates; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type	• Refer data sheet for details about incompatible samples
	• Samples prepared in a different buffer	• Use the reaction buffer provided in the kit or refer data sheet for instructions
	• Samples were not deproteinized (if indicated in datasheet)	• Use the 10 kDa spin cut-off filter or PCA precipitation as indicated
	• Cell/ tissue samples were not completely homogenized	• Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope
	• Samples used after multiple free-thaw cycles	• Aliquot and freeze samples if needed to use multiple times
	• Presence of interfering substance in the sample	• Troubleshoot if needed, deproteinize samples
	• Use of old or inappropriately stored samples	• Use fresh samples or store at correct temperatures till use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components	• Thaw all components completely and mix gently before use
	• Use of expired kit or improperly stored reagents	• Always check the expiry date and store the components appropriately
	• Allowing the reagents to sit for extended times on ice	• Always thaw and prepare fresh reaction mix before use
	• Incorrect incubation times or temperatures	• Refer datasheet & verify correct incubation times and temperatures
	• Incorrect volumes used	• Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components	• Thaw and resuspend all components before preparing the reaction mix
	• Pipetting errors in the standard	• Avoid pipetting small volumes
	• Pipetting errors in the reaction mix	• Prepare a master reaction mix whenever possible
	• Air bubbles formed in well	• Pipette gently against the wall of the tubes
	• Standard stock is at an incorrect concentration	• Always refer the dilutions in the data sheet
	• Calculation errors	• Recheck calculations after referring the data sheet
	• Substituting reagents from older kits/ lots	• Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength	• Check the equipment and the filter setting
	• Samples contain interfering substances	• Troubleshoot if it interferes with the kit
	• Use of incompatible sample type	• Refer data sheet to check if sample is compatible with the kit or optimization is needed
	• Sample readings above/below the linear range	• Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		